Substrate-Attached Glycoproteins from Normal and Virus-Transformed Cells†

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ABSTRACT: Culp and Black (Culp, L. A., and Black, P. H. (1972), *Biochemistry 11*, 2161) have shown that Balb/c 3T3 mouse cells, SV40-transformed 3T3 cells (SVT2), and reverted variants of the transformed cells leave glycoproteins bound to the substrate after their removal with EGTA. We have investigated the nature of this substrate-attached material. The polysaccharide component of the material from both normal and SV40-transformed cells was shown to be quantitatively glycosaminoglycan by its insolubility in solutions of cetylpyridinium chloride (a precipitation assay was developed for glycosaminoglycans using carrier heparin and cetylpyridinium chloride as the precipitating agent), its quantitative breakdown to low molecular weight fragments following treatment with bovine testicular hyaluronidase, and its homogeneous elution with very high salt concentrations from columns of DEAE-Sephadex resin. The glycosaminoglycan is not sulfated, because it eluted from DEAE-Sephadex resin with carrier hyaluronic acid at slightly lower salt concentrations than the sulfated chondroitins and it failed to ncorporate radioactive sulfate. Most of the sulfated glycosaminoglycans produced by normal or transformed cells are secreted into the medium of cultures and not accumulated in the cell fraction. The polysaccharide was shown to be hyaluronic acid by its amino sugar content of glucosamine and its resistance to digestion with chondroitinase ABC under conditions that degrade nonsulfated and sulfated chondroitins to $\Delta^{4,5}$-unsaturated disaccharides. Filtration through Sepharose gels revealed at least two size classes, both containing protein and polysaccharide in ratios that differed between normal and transformed cells. Pronase digestion resulted in three size classes of polysaccharide—two with molecular weights of 10^5–10^6 and a third of smaller size (still >10,000). Hyaluronidase digestion liberated two size classes of polypeptide; the minor portion was excluded from Sephadex G-50 resin, while the major portion was much smaller in size. The small polypeptide material may be covalently linked to the hyaluronic acid polysaccharide chains. The molecular composition of substrate-attached glycoproteins from normal and virus-transformed cells suggests a role for their involvement in cell-to-substrate adhesion, and possibly cell-to-cell adhesion.

Normal and virus-transformed mouse fibroblasts adhere to a solid substrate for growth in culture. This establishes a unique interface between the cell surface and the surface of this substrate, and the study of this interaction may prove valuable for predicting how cells interact. Gail and Boone (1972) have shown that transformed cells adhere less tenaciously to the substrate than do normal cells. Willis (1967) has emphasized the importance of reduced adhesiveness by malignant cells to permit dissociation of cells from a tumor mass and metastasis to a second site in the body, where the adhesive specificity between the malignant and the normal cells at the implant site permits the malignant cells to (a) attach and then (b) grow into a tumor mass. Much evidence (Black *et al.*, 1971) implicates alterations in the cell surface for loss of contact inhibition of growth upon viral transformation. Study of the cell–substrate interface may provide valuable information as to the mechanisms of these phenomena.

Culp and Black (1972b) used chelating agents such as EDTA[1] (for Mg^{2+} and Ca^{2+} chelation) or EGTA (specifically for Ca^{2+} chelation) to remove Balb/c mouse 3T3 fibroblasts or Simian virus 40 (SV40) transformed fibroblasts from the substrate (Falcon plastic or Brockway glass). They found that the surface of the substrate, although free of any cells, vesicles, or organelles, was covered with protein- and polysaccharide-containing macromolecules. The amounts of this substrate-attached material (SAM) correlated with the flat, highly spread morphology of Balb/c 3T3 cells and the concanavalin A selected revertant cells of the SV40-transformed cells; *i.e.*, these flat, contact-inhibited cell lines deposited three to six times more of this material than the smaller, spindle-shaped transformed cells. This evidence plus the fact that SAM was so tenaciously bound to the substrate, requiring a somewhat lengthy treatment with strong alkali or ionic detergents to remove it quantitatively, suggested that this material may be important in cell-to-substrate attachment.

This study expands the analysis of this SAM to determine if there are qualitative differences, as well as quantitative differences, in the SAM deposited by Balb/c 3T3 cells, SV40-transformed 3T3 cells, or Con A selected reverted variants of transformed cells (Culp and Black, 1972a); revertant cells have regained the flat morphology and contact-inhibitory properties of normal cells, while retaining the complete SV40 genome in its repressed form (Pollack *et al.*, 1968; Culp *et al.*, 1971; Culp and Black, 1972a). The nature of the protein and polysaccharide portions of SAM has been examined in some detail.

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[1] Abbreviations used are: BD, Blue Dextran; Con A, concanavalin A; CPC, cetylpyridinium chloride; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; GAG, acidic glycosaminoglycans (formerly referred to as mucopolysaccharides); lmw, low molecular weight marker; MEM×4, Eagle's minimal essential medium supplemented with four times the concentration of vitamins and amino acids; PBS, phosphate-buffered saline; SAM, substrate-attached material; SV40, Simian virus 40.

Materials and Methods

Cell Growth. Mouse fibroblast Balb/c 3T3 cells (clone A31) and SV40-transformed A31 cells (clone SVT2) were obtained from Dr. Stuart Aaronson (National Cancer Institute, NIH) after 150 generations of growth and were used in this laboratory between their 6th and 18th passages (a passage is approximately four generations of growth). Concanavalin A (Con A) selected revertant cells of SVT2 (revertant clone 84) which have been described by Culp and Black (1972a) were used between their 9th and 15th passages. All cells were grown in Eagles' minimal essential medium (MEM×4) supplemented with four times the concentration of amino acids and vitamins, 10% fetal calf serum, penicillin (250 units/ml), and streptomycin (0.25 mg/ml). Cells were incubated in an environment of 5% CO_2 in air which was humidified and maintained at 37°. Cells were routinely passaged in Brockway glass 32-oz. tissue culture bottles by using a trypsin–EDTA solution.

All cells used for experimentation were *Mycoplasma* free according to the radiolabel assay of Culp and Black (1972b).

Radiolabeling Procedures. (A) FOR SUBSTRATE-ATTACHED MATERIAL ISOLATION. Trypsinized cells were centrifuged at 225*g* for 10 min and resuspended in trypsin-free medium. The cells were inoculated at the following concentrations into 100-mm diameter Falcon tissue culture dishes containing 6 ml of medium with a radioactive precursor: 3T3, 0.5×10^6 cells; SVT2, 1.25×10^6 cells; and Con A revertant, 0.4×10^6 cells/dish. Medium for D-glucosamine radiolabeling contained MEM×4 with 2.5–4.0 μCi/ml of D-glucosamine [1-^{3}H]hydrochloride (specific activity 1.1 Ci/mmol) or 0.5–1.0 μCi/ml of D-glucosamine [1-^{14}C]hydrochloride (specific activity 56 Ci/mol). For L-leucine radiolabeling, leucine-free MEM×4 supplemented with 3 mg/l. of L-leucine was used to maintain a high specific radioactivity of leucine in this medium. The concentration of L-[4,5-^{3}H]leucine was 2.0–4.0 μCi/ml (specific activity 41.2 Ci/mmol).

(B) FOR CPC ASSAY. Trypsinized cells were inoculated into triplicate 60-mm diameter Falcon plastic tissue culture dishes for each datum point. Inoculated cell densities were: 0.25×10^6 3T3 cells or 0.2×10^6 SVT2 cells per dish. Four milliliters of radiolabel medium (2.0–3.0 μCi/ml of D-[^{3}H]glucosamine or 4.0–5.0 μCi/ml of $Na_2{}^{35}SO_4$ (specific activity 500–1000 Ci/mol)) was added to two dishes. Control dishes were used to determine cell number.

Isolation of Cell Fractions. Cultures[2] were divided into medium, cell, and substrate-attached fractions in the following manner (Culp and Black, 1972b). After incubation of cells under the appropriate conditions, medium was decanted and saved; the cell layer was then rinsed three times with PBS. EGTA (0.5 mM in PBS) was added to the cell culture (2.0 ml/60-mm dish and 4.0 ml/100-mm culture dish). The cells were incubated on a rotary shaker at 37° for 30 min, and the suspension of cells was gently pipetted to remove any attached cells. Distilled water was utilized to rinse the substrate thoroughly. The SAM fraction was removed with either 0.1% sodium dodecyl sulfate or 0.1 N NaOH in the same volume as the chelating agent by rotation on a shaker at 37° for 30 min (Culp and Black, 1972b); these extraction conditions did not appear to affect the properties of SAM established in these studies. These concentrations of reagents were found to remove the substrate-attached material quantitatively.

[2] All cultures for experimental purposes were grown on Falcon plastic tissue culture dishes.

For isolation of SAM for chromatography the substrate-attached fraction from 100-mm culture dishes was extracted with 0.1 N NaOH. The alkaline solution was neutralized with HCl to pH 7 and dialyzed against 50 volumes of 0.01 M sodium phosphate buffer at pH 7 for 24–48 hr to reduce the salt concentration. The material was then lyophilized to dryness and redissolved in 1.5 ml of 0.01 M sodium phosphate buffer for enzymatic digestions and chromatography.

Precipitation Assays. Aliquots of cell (0.1 ml), medium (20 μl), and substrate-attached (0.1 ml) fractions were brought to a final volume of 0.5 ml by addition of distilled water. To assay for cetylpyridinium chloride (CPC) precipitable radioactivity, sodium heparin (60–100 μg) was used as carrier glycosaminoglycan and CPC was added at three times the heparin concentration. The reaction mix was allowed to incubate at 25° for 30 min, and the precipitates were collected on Millipore filters having a 0.45 μ pore size. The Millipore filters were dried, and their radioactive content was determined using a Packard scintillation counter.

The samples were also assayed by making them 5% (w/v) CCl_3COOH after the addition of 100 μg of carrier bovine serum albumin to precipitate proteins, glycoproteins, and some glycosaminoglycans (Kraemer, 1971a,b). Precipitation occurred during incubation in the cold (0–4°) for 30 min. To determine the amount of CCl_3COOH-precipitable radioactivity, the fractions were filtered through Whatman 2.4-cm GF/C glass fiber filters which were assayed for their radioactivity in the same manner as the Millipore filters.

Fractionation. A. GEL FILTRATION. All columns were 1.2 cm in diameter and approximately 55 cm in length. Sephadex G-50 and G-200 and Sepharose 6B gels were swollen as recommended by the manufacturer in the buffer used to elute the column (0.1 M sodium phosphate (pH 7.0) containing 0.1% sodium dodecyl sulfate). Fractions of 1 ml were collected and 0.5-ml aliquots were assayed for radioactivity in Bray's scintillation fluid (Bray, 1960). Blue Dextran (BD) and radiolabeled thymidine (lmw) were used for high and low molecular weight markers, respectively; 90–100% of the radioactive material applied to columns was eluted.

B. ION-EXCHANGE CHROMATOGRAPHY. DEAE-Sephadex A-25 (Cl^- form) was swollen in dilute HCl and equilibrated with 0.05 M sodium phosphate buffer (pH 7.0), and 0.5 ml of each 2.0-ml fraction was assayed for its radioactive content in Bray's scintillation fluid.

Hexosamine determinations of acid-hydrolyzed materials were done on Dowex 50-X8 (hydrogen form) columns (1.2 × 55 cm). The sample, with carrier hexosamine (D-glucosamine and D-galactosamine), was applied in water and eluted with 0.3 N HCl.

C. PAPER CHROMATOGRAPHY. Enzyme-digested samples were dried on Whatman No. 1 chromatography paper and desalted with a solvent of butanol–ethanol–water (52:32:16). After 40-hr desalting, the chromatogram was placed in a second solvent system for 48 hr containing butyric acid and 0.5 N NH_4OH (5:3). Disaccharides resulting from chondroitinase ABC digestion are $\Delta^{4,5}$ unsaturated and can be observed with the use of ultraviolet light (Yamagata *et al.*, 1968). The radioactively labeled SAM digestion products were assayed by cutting the chromatogram into 4-mm wide strips, eluting each overnight with 0.5 ml of water, and assaying for radioactive content using Bray's scintillation fluid.

Sugar Analysis. A. ACID HYDROLYSIS PRODUCTS. Hexosamine determinations were performed on radiolabeled SAM which had been hydrolyzed with 2 N trifluoroacetic acid (Grimes, 1973).

B. Uronic Acid Assay. Carrier glycosaminoglycans were assayed according to the modified carbazole method (Bitter and Muir, 1962).

C. Hexosamine Determinations. Carrier hexosamines were determined by the Elson–Morgan method (Elson and Morgan, 1933).

Enzymatic Treatments of Cell Fractions. Digestions of glycosaminoglycans with hyaluronidase were done in 0.01 M sodium phosphate buffer (pH 6.0–6.6). The digestion mixture contained 0.4–1.0 ml of concentrated SAM (4000–9000 cpm) in buffer, bovine testicular hyaluronidase (700–860 units), and three drops of toluene to assure sterility. The samples were incubated for 72 hr in a 37° water bath. Pronase digestions of protein-containing fractions were done under the same conditions as described above, except that hyaluronidase was replaced with 1.0 mg of Pronase and the final mixture was brought to 5 mM with respect to $CaCl_2$. "Self-digestions" were performed at the same time, but omitting the enzyme from the digestion mixtures. Subsequent to digestion the samples were made 0.1% in sodium dodecyl sulfate and chromatographed.

Digestions of sulfated and nonsulfated chondroitins were performed with chondroitinase ABC as described by Yamagata *et al.* (1968) in the following manner: digestion mixtures contained either 0.4 ml of radiolabeled SAM (after dialysis against Tris buffer containing 24 g/l. of sodium acetate, 14.6 g/l. of NaCl, 500 mg/l. of bovine serum albumin, and 30 g/l. of tris(hydroxymethyl)aminomethane for 24 hr) plus 200 μg of hyaluronate or 200 μg of another glycosaminoglycan (hyaluronic acid or sulfated and nonsulfated chondroitins) as a control. At the beginning of the 37° incubation 0.1 unit of enzyme was added to each tube, and again at the midpoint of the 6-hr digestion 0.1 unit of enzyme was added. Digested samples from these incubations were dried and chromatographed on Whatman No. 1 paper.

Analysis of Radioactivity. Both ^3H:^{14}C and ^3H:^{35}S double radiolabeling was used. In all cases of significant spillover the appropriate values were subtracted from the gross tritium-channel data to give the net tritium data values.

Materials were purchased from the following sources: D-[1-^{14}C]glucosamine from Amersham/Searle and New England Nuclear Corp.; D-[1-^{3}H]glucosamine from Amersham/Searle and Schwarz/Mann; L-[4,5-^{3}H(N)]leucine from New England Nuclear; trifluoroacetic acid, EDTA, and EGTA from Eastman Organic Chemicals; cetylpyridinium chloride (hexadecylpyridinium chloride), bovine albumin (fraction V), hyaluronic acid (grade III), and chondroitin sulfate (mixed) from Sigma Chemical Co.; Sephadex G-50 gel, Sepharose 6B gel, and DEAE-Sephadex A-25 resin from Pharmacia Fine Chemicals Inc.; Dowex 50-X8 resin (fine mesh) from Bio-Rad Laboratories; MEM×4 and fetal calf serum from Grand Island Biologicals Co.; plastic tissue culture dishes from Falcon Plastics; 32-oz Brockway glass prescription bottles from Brockway Glass Inc.; Millipore membranes (25 mm, HAWP, 0.45 μ pore size) from Millipore Corp.; Whatman No. 1 chromatography paper and Whatman glass fiber filters (GF/C) from Arthur H. Thomas Co.; testicular hyaluronidase (chromatographically pure) from Worthington Biochemical Corp.; Pronase (grade B) from Calbiochem Corp.; sodium heparin (injectable) from Upjohn Co. Chondroitinase ABC (Miles Laboratories Research Division), 2-acetamido-2-deoxy-3-*O*-(Δ-D-glucos-4-enepyranosyluronic acid)-D-galactose (Miles Laboratories Research Division), and purified human umbilical cord hyaluronate were the generous gifts of Dr. Irwin Schafer.

Results

Culp and Black (1972b) found a quantitative correlation among (1) the amounts of substrate-attached material (SAM) deposited on the substrate, (2) the contact-induced inhibition of growth, and (3) the flat, highly spread morphology of these cells. The question as to whether or not there existed a qualitative difference between the SAM of these cell lines remained unanswered.

Since the SAM could be very readily radiolabeled with glucosamine, and therefore possessed large amounts of carbohydrate, it may be composed partially of glycosaminoglycans (GAG), which are not quantitatively precipitated with CCl_3COOH (Kraemer, 1971a,b). An assay procedure to determine the amount of GAG present was therefore needed. It has been known that cetylpyridinium chloride (CPC) precipitates such negatively charged macromolecules as GAG, and this procedure has been used in a number of biological systems to purify GAG from other cell components (Toole and Trelstad, 1971).

By using this CPC precipitation procedure (described in the Materials and Methods section) it was found that there was more radioactive glycoprotein insoluble in CPC than in CCl_3COOH from the medium and SAM fractions of the cell. One milliliter of conditioned medium (medium removed from cell cultures subsequent to cell growth in radioactive glucosamine) was CCl_3COOH precipitated, and the soluble fraction was neutralized and dialyzed to remove the remaining CCl_3COOH. This dialysate was then precipitated with either CPC or reprecipitated with CCl_3COOH. It was found that 1 ml of this glucosamine-radiolabeled dialysate yielded 52% more radioactivity upon CPC precipitation while neglible radioactivity was filterable after a second CCl_3COOH precipitation. Thus, a major portion of glucosamine-radiolabeled material from conditioned medium is presumably GAG which is not readily CCl_3COOH precipitable.

To determine the efficiency of CPC precipitation of GAG, conditioned medium from $Na^{35}SO_4$-radiolabeled cell cultures was utilized, because essentially all macromolecules which contain ^{35}S covalently bound are GAG and should presumably be insoluble in CPC (Kraemer, 1971c). The filtrate of 1 ml of such medium after precipitation with CPC was made 0.1% in sodium dodecyl sulfate to prevent any aggregation and chromatographed on Sephadex G-50 to determine if all macromolecules containing radioactive sulfate are trapped on the filter. Figure 1A illustrates that only a peak of low molecular weight radioactivity was found. Such a peak should correspond to unincorporated radioactive sulfate in this culture medium. The complementary experiment was performed to determine if all radioactivity precipitated is of high molecular weight. The CPC-generated precipitate was collected by centrifugation at 6400*g* for 30 min and washed with a dilute CPC (0.1 μg/ml) solution three times. The pellet was redissolved in 0.1% sodium dodecyl sulfate to dissociate aggregates at 37° and chromatographed on a Sephadex G-50 column. The resulting profile of eluted radioactivity is shown in Figure 1B. There was a large excluded peak of radioactivity which represented macromolecular components and eluted with a Blue Dextran marker, and also a smaller radioactive peak, probably indicating a small amount of coprecipitation of unincorporated sulfate precursor. The macromolecular peak contained 71.4% of the total radioactivity eluted from the column, while the low molecular weight peak represented 28.6%.

Medium, cell, and substrate-attached fractions from both

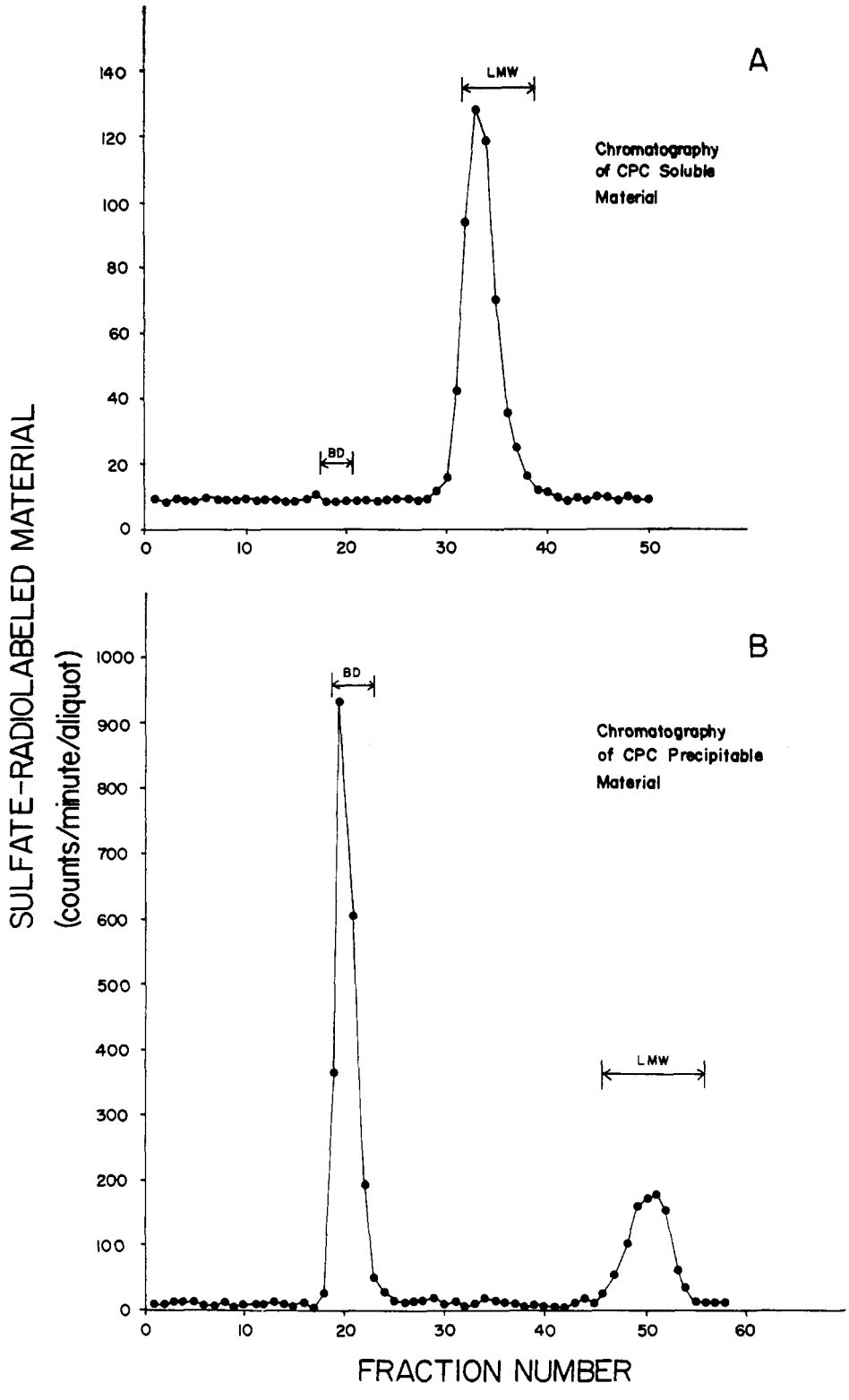

FIGURE 1: Chromatography of CPC-soluble and -insoluble material from conditioned medium. Radiolabeled conditioned medium from 3T3 cells which had been grown for 48 hr in medium containing $Na_2{}^{34}SO_4$ was precipitated with CPC. Chromatography of these samples (A and B) was performed on Sephadex G-50 resin with a 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate. Samples of 1 ml were collected from the 1.2 × 55 cm columns (flow rate of approximately 30 ml/hr); 0.5-ml aliquots were assayed for radioactivity in Bray's scintillation fluid. CPC precipitation was accomplished in the following manner: 0.2 ml of conditioned medium was brought up to a final volume of 0.5 ml with distilled water and then incubated for 30 min after the addition of 100 μg of carrier heparin and 300 μg of cetylpyridinium chloride. (A) Twenty microliters of medium were precipitated with CPC as described in the Materials and Methods section. The incubation mixture was filtered through a Millipore filter with a 0.45 μ pore size, and the filtrate was collected for chromatography. (B) The CPC-insoluble material was separated by centrifugation at 6400*g* for 30 min and the pellet was then redissolved in 0.1% sodium dodecyl sulfate for chromatography.

normal and SV40-transformed cell cultures were assayed for CPC-precipitable material (Table I). As can be seen from this table there was a class of CPC-precipitable molecules in each of the three fractions from both cell lines, indicating

TABLE I: CPC Precipitation of [^{3}H]Glucosamine-Radiolabeled Cell Culture Fractions.[a]

	3T3[b]	SVT2[b]
Medium	49,000	105,000
Cell	65,000	120,000
SAM	2,300	2,100

[a] Cells were inoculated into 60-mm culture dishes at the following concentrations: 0.25 $\times$ 10^6 cells/dish for 3T3 cells and 0.20 $\times$ 10^6 cells/dish for SVT2 cells. The medium was replaced 24 hr later with MEM$\times$4 containing 2.5 μCi/ml of [^{3}H]glucosamine. The cell cultures were fractionated into medium, cell, and substrate-attached fractions as described in the Materials and Methods after 48-hr growth. The culture fractions were precipitated with CPC as described in the Materials and Methods section. [b] Radioactive content (cpm) per dish.

that each fraction possessed some glycosaminoglycan. All of the radioactivity in the SAM fraction was CPC precipitable (unpublished data).

To determine the molecular nature of the substrate-attached material, it was isolated in large quantities by the methods described previously (Culp and Black, 1972b, and the Materials and Methods section), and its composition was examined. Since SAM was shown to be CPC precipitable, it was important to determine by other criteria the content of glycosaminoglycans. This was accomplished by the use of bovine testicular hyaluronidase, which will specifically degrade the nonsulfated glycosaminoglycans chondroitin and

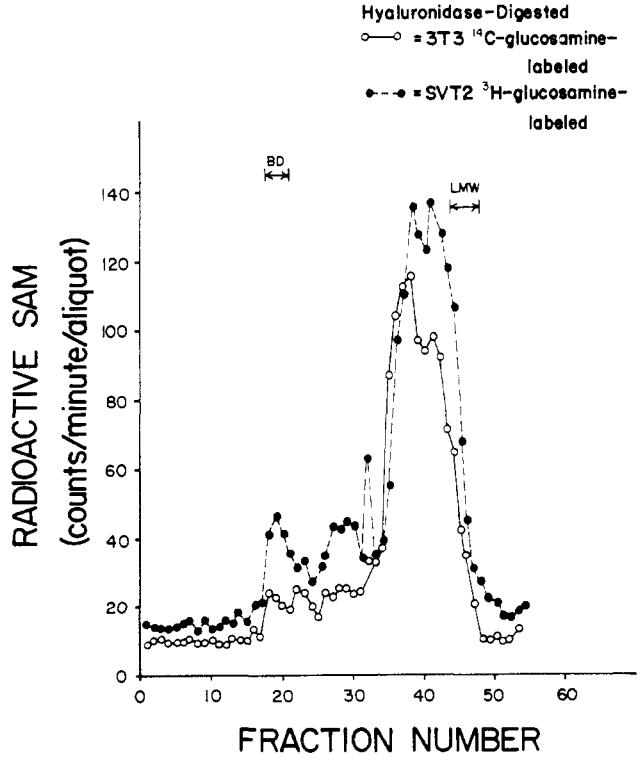

FIGURE 2: Hyaluronidase digestion of glucosamine-radiolabeled SAM from 3T3 and SVT2 cells. Sephadex G-50 chromatography following hyaluronidase digestions of SAM was performed as described in the Materials and Methods section and Figure 1.

TABLE II: Distribution of Insoluble Glycoprotein and Sulfated Glycosaminoglycan in 3T3 and SVT2 Cell Cultures.[a]

	Radioactive Content/Fraction (%)[b]			
	[^{3}H]Glucosamine Incorp		$^{35}SO_4^{2-}$ Incorp	
Culture Fractions	CPC[c]	CCl_3-COOH[c]	CPC[c]	CCl_3-COOH[c]
	3T3			
Medium	929,000	299,000	6,126,000	593,000
Cell	92,860	94,500	26,700	29,340
Substrate attached	5,420	5,860	2,480	3,160
	SVT2			
Medium	1,347,000	475,000	6,528,000	63,750
Cell	637,800	378,400	30,000	23,900
Substrate attached	9,440	10,540	732	722

[a] Cells were inoculated into five 60-mm culture dishes (3T3, 0.25 $\times$ 10^6 cells/dish; SVT2, 1.2 $\times$ 10^6 cells/dish); 24 hr later the medium was changed to medium containing radioactive precursors (20 μCi/ml of $Na_2{}^{35}SO_4$; 4.0 μCi/ml of [^{3}H]glucosamine). The cells were allowed to grow from the initial density to approximately 50% confluency by 48 hr (0.75 $\times$ 10^6 3T3 cells/dish or 3.43 $\times$ 10^6 SVT2 cells/dish); the cell cultures were then fractionated into cell, medium, and substrate-attached fractions as has been described in the Materials and Methods section. [b] Each value represents the mean of ten measurements and indicates total amount of radioactivity in each fraction per culture dish. [c] This reagent was used as the precipitating agent: CPC for GAG or CCl_3-COOH for glycoproteins and some GAG.

hyaluronic acid and the sulfated chondroitins A and C, but not the heparin sulfates (Ludoweig *et al.*, 1961). Incubation of hyaluronidase with leucine-radiolabeled soluble cell proteins or nonradioactive hyaluronic acid at 37° for 48 hr did not result in any extensive proteolysis of cell proteins but did result in extensive hyaluronate breakdown to small fragments as determined by Sephadex G-50 chromatography (unpublished data). Thus hyaluronidase preparations were efficient in degrading GAG polysaccharide under conditions whereby minimal breakdown of the protein portion of glycoproteins resulted.

Glucosamine-radiolabeled SAM from both 3T3 and SVT2 cells were treated with this hyaluronidase preparation and chromatographed on Sephadex G-50 columns. Figure 2 shows that the polysaccharide components of both SAM fractions have been reduced in molecular size almost quantitatively. Control digestions were done under the same conditions with doubly radiolabeled ([^{14}C]glucosamine and [^{3}H]leucine) SAM omitting only the enzyme with no resultant degradation. Therefore SAM from both 3T3 and SVT2 cells was highly sensitive to bovine testicular hyaluronidase.

To determine whether the GAG in SAM was sulfated, cells were grown in medium containing [^{3}H]glucosamine and $Na_2{}^{35}SO_4$ for 48 hr, followed by fractionation of the cultures into cell, medium, and substrate-attached fractions. The radioactive GAG was assayed by CPC or CCl_3COOH precipitation, and Table II presents the data obtained by this

TABLE III: Comparison of Insoluble Glycoprotein and Sulfated Glycosaminoglycan in 3T3 and SVT2 Cell Cultures.[a]

Culture Fraction	Radioactive Content/Fraction[b] (%)			
	[^{3}H]Glucosamine Incorp		$^{35}SO_4^{2-}$ Incorp	
	CPC	CCl_3COOH	CPC	CCl_3COOH
	3T3			
Medium	90.4	74.9	99.5	94.8
Cell	9.0	23.6	0.43	4.7
Substrate attached	0.527	1.46	0.04	0.505
Total	100	100	100	100
	SVT2			
Medium	67.5	55.0	99.5	72.1
Cell	32.0	43.8	0.45	27.0
Substrate attached	0.473	1.2	0.011	0.81
Total	100	100	100	100

[a] These data are expressions of the values from Table II of the relative percentages of glycoprotein (including GAG) of the entire culture for 3T3 and SVT2 cells. See Table II for procedures of cell growth, radiolabeling, and assay procedures. [b] The percentage values were obtained by the following equation.

$$\frac{\text{Radioactivity}_{\text{particular fraction}}}{\text{Radioactivity}_{(\text{cell + medium + SAM})}} \times 100 = \%$$

procedure for each fraction. When the percentage of total CPC-insoluble radioactivity was determined (Table III), it could be seen that there were only negligible amounts of ^{35}S radioactivity in the substrate fraction. Almost all (99.5%) of the ^{35}S-radiolabeled material which was CPC or CCl_3COOH insoluble was found in the medium fraction, and only a small percentage was discovered in the cell fraction. These observations were true whether cells were grown for short or long periods in radioactive precursors. These results indicated that the hyaluronidase-susceptible, CPC-insoluble component of the SAM was nonsulfated, and was therefore either hyaluronic acid or nonsulfated chondroitin. These experiments also indicated that essentially all sulfated glycosaminoglycans were secreted into the medium and that a pool of these materials did not accumulate within the cell.

Since glycosaminoglycans are highly negatively charged (being CPC precipitable), it was expedient to investigate the charge homogeneity of SAM materials by using DEAE-Sephadex A-25 chromatography of radiolabeled SAM. Glucosamine-radiolabeled SAM from both 3T3 and SVT2 cells were chromatographed with carrier GAG (hyaluronate, chondroitin sulfate, and sodium heparin (also sulfated)). As can be seen in Figure 3 both 3T3 and SVT2 SAM eluted almost quantitatively at 0.85 M NaCl. No glycoprotein was eluted at low salt concentrations, indicating that all of the polysaccharide of the SAM preparations was GAG. Carrier hyaluronate also eluted at 0.85 M NaCl, while chondroitin sulfate and sodium heparin eluted at 1.3 M NaCl from the same column (the elution range of these GAG's were determined separately). These results again indicated SAM glycosaminoglycan was a nonsulfated species.

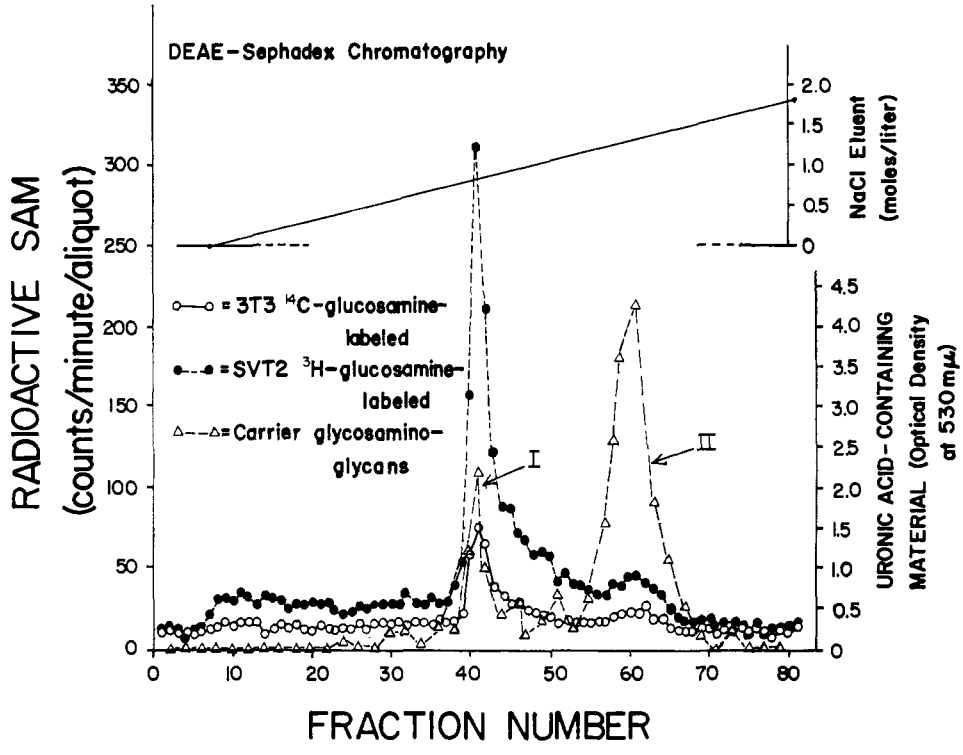

FIGURE 3: DEAE-Sephadex chromatography of glucosamine-radiolabeled SAM from 3T3 and SVT2 cells. Glucosamine-radiolabeled SAM was isolated as described in the Materials and Methods section from exponentially growing cells and chromatographed on a DEAE-Sephadex A-25 column. Fractions of 2 ml were collected from the 1.2 × 55 cm column, which had a flow rate of approximately 3 ml/hr. Carrier GAG's were hyaluronic acid (1.0 mg), chondroitin sulfate (3.0 mg), and sodium heparin (9.0 mg). The radioactivity of each fraction was determined as in Figure 1, and the uronic acid containing material was assayed by the modified carbazole method as described in the Materials and Methods section. Other DEAE-Sephadex chromatography (unpublished data) has shown that the hyaluronate elutes in the peak I region, while chondroitin sulfate and heparin elute in the region of peak II.

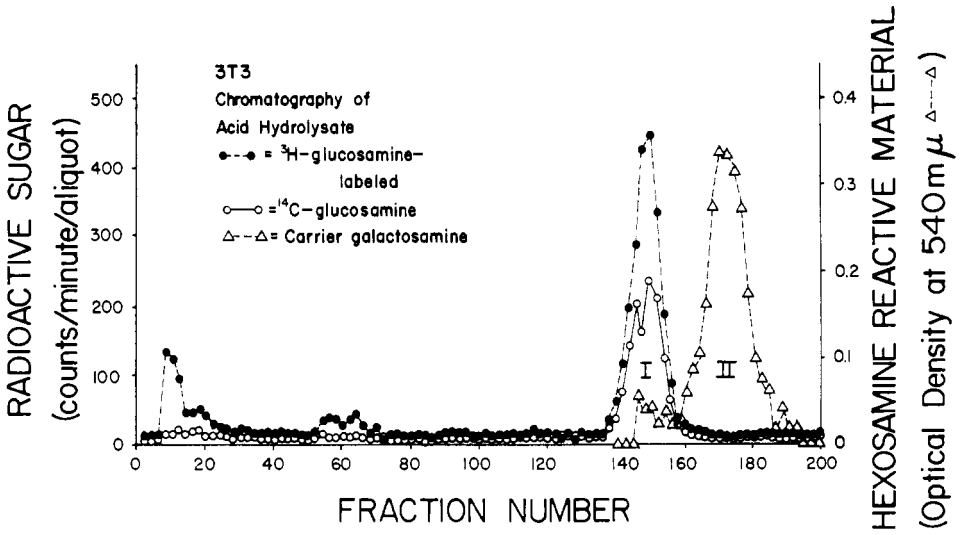

FIGURE 4: Dowex chromatography of acid-hydrolyzed glucosamine-radiolabeled SAM from 3T3 cells. [^{3}H]Glucosamine-radiolabeled SAM was hydrolyzed by trifluoroacetic acid (as described in the Materials and Methods section), and the resulting hydrolysate was chromatographed with 10.0 μmol of carrier galactosamine and 3500 dpm of [^{14}C]glucosamine on Dowex 50-X8 (1 $\times$ 50 cm). Fractions of 2 ml were collected at the rate of 13 ml/hr. The radioactive analysis of fractions was as described in Figure 2, and the hexosamine determinations were performed on 1.0-ml aliquots of fractions by the Elson–Morgan reaction as described in the Materials and Methods section.

Although the results using ion exchange columns indicate that the GAG in SAM eluted with hyaluronic acid, it was still possible that the glycosaminoglycan could be nonsulfated chondroitin. The amino sugar composition of GAG is specific; chondroitins contain *N*-acetylgalactosamine, while *N*-acetylglucosamine is found in hyaluronate. Glucosamine-radiolabeled SAM from 3T3 and SVT2 cell was hydrolyzed with trifluoroacetic acid and chromatographed on Dowex 50 resin to separate amino sugars. Figures 4 and 5 give the elution profiles of the SAM hydrolysates and carrier amino sugars. The [^{3}H]glucosamine-radiolabeled hydrolysates from both 3T3 and SVT2 cells were shown to elute with radiolabeled and unlabeled glucosamine and separately from carrier galactosamine. Since the amino sugar component of SAM is glucosamine, the GAG in the substrate-attached material has been therefore identified as hyaluronic acid.

As another line of supportive evidence, enzyme digestion with chondroitinase ABC was performed. Chondroitinase ABC is an enzyme which will readily digest most GAG, except hyaluronate, into $\Delta^{4,5}$-unsaturated disccharides specific to the various classes of glycosaminoglycans. Figure 6 illustrates the chromatographic results of chondroitinase ABC treatment of glucosamine-radiolabeled SAM from 3T3 cells. There was no digestion of SAM as determined by the persistence of radioactivity on the origin of the chromatogram. Carrier chondroitin, however, was degraded under these same conditions while carrier hyaluronate was not digested. Identical results were observed after attempted digestion of SAM from SVT2 cells.

After the identification of the glycosaminoglycan portion of the substrate-attached glycoprotein it was important to investigate the structural composition of the carbohydrate and protein portions of this material. Utilizing 0.1% sodium dodecyl sulfate or 0.1 N NaOH (at 4°) for deaggregation, the isolated SAM was chromatographed on Sephadex G-50 resin after radiolabeling with [^{14}C]glucosamine and [^{3}H]leu-

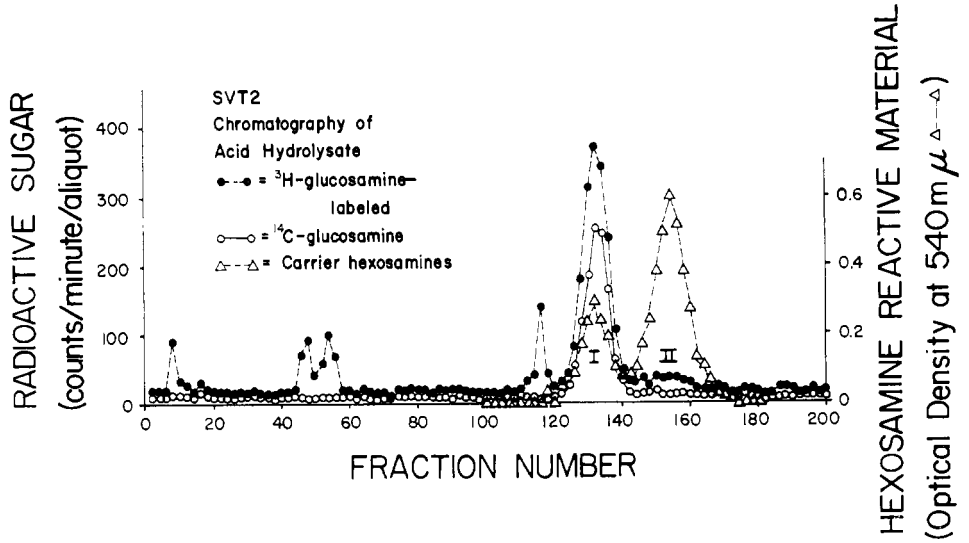

FIGURE 5: Dowex chromatography of acid-hydrolyzed glucosamine-radiolabeled SAM from SVT2 cells. The treatment of the [^{3}H]glucosamine-radiolabeled SAM from SVT2 cells was the same as described in Figure 4, with the exception that 3.0 μmol of carrier glucosamine was also chromatographed with the markers mentioned in Figure 4.

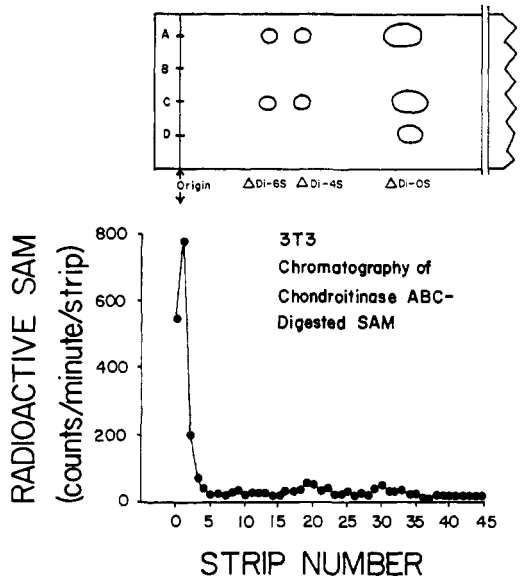

FIGURE 6: Paper chromatography of chondroitinase ABC digested SAM from 3T3 cells. SAM from 3T3 cells grown in medium containing [^{3}H]glucosamine was digested with the enzyme preparation chondroitinase ABC and the following digestion mixtures were dried on the origin of the chromatogram: (A) 0.4 ml of radiolabeled SAM and 200 μg of chondroitin (in the same incubation mixture) after digestion with 0.2 unit of chondroitinase ABC at 37° for 5 hr; (B) 100 μg of purified hyaluronic acid after digestion with 0.2 unit of chondroitinase ABC as described in (A); (C) 200 μg of chondroitin after digestion with 0.2 unit of chondroitinase ABC as described in (A); and (D) 30 μg of ΔDi-OS (2-acetamido-2-deoxy-3-*O*-(Δ^{4,5}-D-glucos-4-enepyranosyluronic acid)-D-galactose, the Δ^{4,5}-unsaturated disaccharide resulting from chondroitinase ABC digestion of nonsulfated chondroitin) was applied as a known marker. The chromatography systems and the determination of radioactivity have been described in the Materials and Methods section. ΔDi-6S and ΔDi-4S indicate the disaccharides from chondroitin 6-sulfate and chondroitin 4-sulfate digestion, respectively, with chondroitinase ABC.

cine, to determine if this material contained any low molecular weight constituents. SAM from both 3T3 and SVT2 cells was found to be quantitatively excluded from this resin (unpublished data), indicating that all components had a molecular size greater than that of the exclusion limit of the resin (>50,000 daltons of protein or 10,000 daltons of polysaccharide). In an effort to measure the molecular size of the SAM, Sepharose 6B resin with a polysaccharide exclusion limit of one million daltons and a polypeptide exclusion limit of four million daltons was utilized. Figures 7 and 8 show the profiles of radioactivity from doubly radiolabeled ([^{14}C]-glucosamine and [^{3}H]leucine) SAM from 3T3 and SVT2 cells, respectively. In all cases each profile contained an excluded peak of radioactivity (peak I) which eluted concurrently with the beginning of the blue dextran high molecular weight marker. This excluded peak of radioactivity contained both radiolabeled glucosamine and leucine. Each chromatograph also showed one major included peak of radioactivity (peak II), corresponding to carbohydrate- and protein-containing molecules of less than one million molecular weight. The third peak of radioactivity (peak III) seen in the SVT2 profile varied in size each time the experiment was repeated and may be the result of breakdown of larger molecules through handling of the SAM fraction or contamination with simple glycoproteins. The profiles of elution of SAM from the Con A revertant cells were similar to those in Figures 7 and 8 (unpublished data). SAM from all three cell

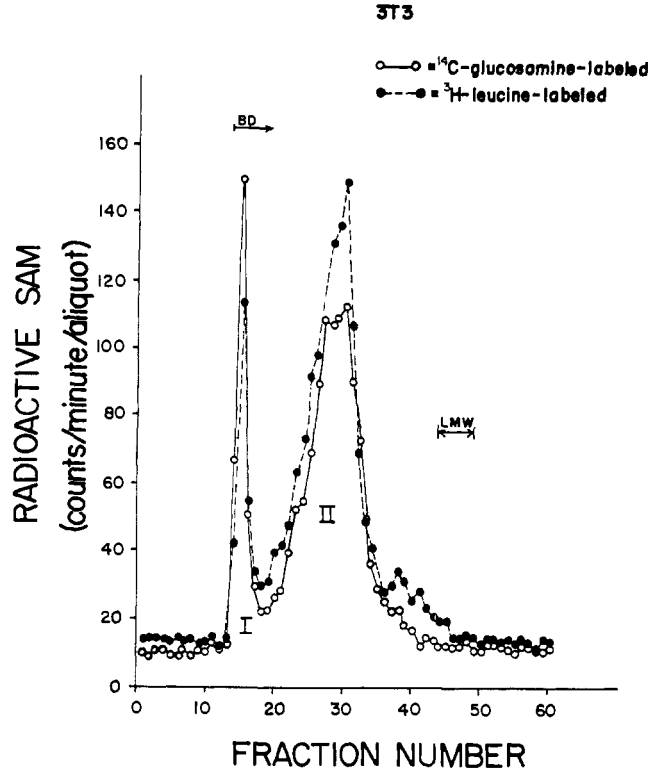

FIGURE 7: Sepharose 6B chromatography of SAM from 3T3 cells. [^{14}C]Glucosamine- and [^{3}H]leucine-radiolabeled SAM was isolated as described in the Materials and Methods section. Sepharose 6B chromatography (on a 1 × 50 cm column) was performed using a 5–6 ml/hr flow rate. The fraction size was 1.0 ml, of which 0.5 ml was assayed for its radioactivity as described in Figure 1.

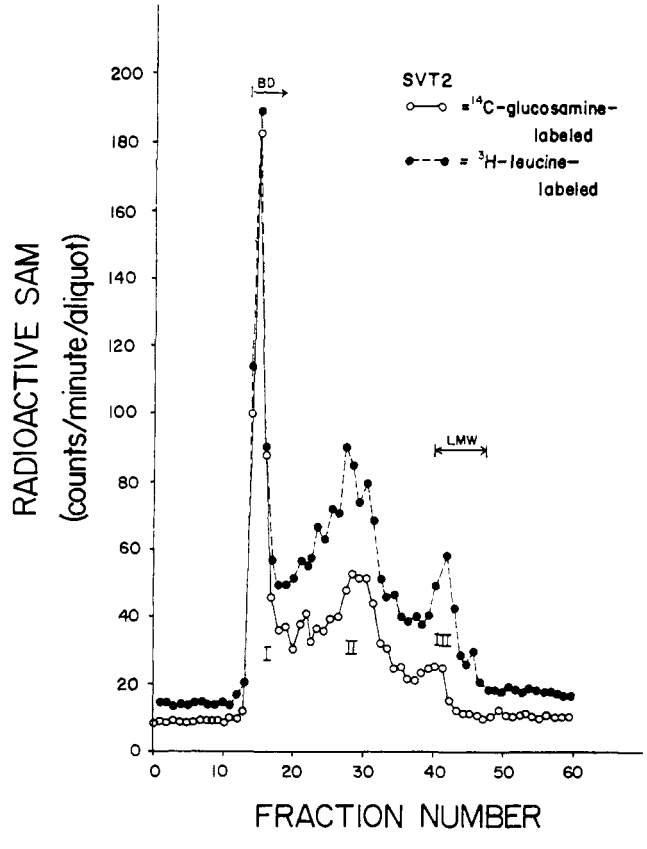

FIGURE 8: Sepharose 6B chromatography of SAM from SVT2 cells. See Figure 7 for procedures used. Cells were grown and radiolabeled during exponential growth.

TABLE IV: Distribution of SAM Radioactivity from 3T3, SVT2, and Revertant Cell Cultures after Sepharose Chromatography.[a]

Cell Type	Radioactive Precursor	Peak I (%)	Peak II (%)	Total[b] (%)
3T3	[^{14}C]-Glucosamine	22.8	77.2	100
	[^{3}H]Leucine	12.9	87.1	100
	^{14}C:^{3}H[c]	1.307	0.657	
SVT2	[^{14}C]-Glucosamine	43.2	56.8	100
	[^{3}H]Leucine	33.0	67.0	100
	^{14}C:^{3}H[c]	0.983	0.635	
Con A revertant	[^{14}C]-Glucosamine	39.1	60.9	100
	[^{3}H]Leucine	54.6	45.4	100
	^{14}C:^{3}H[c]	0.408	0.767	

[a] The data refer to the recovery (in percentages) of radiolabeled material as displayed in Figures 7 and 8 for 3T3 and SVT2 cells and unpublished data for the Con A revertant cells. Peak I indicates the excluded peak of radioactivity in these figures, while peak II represents the included radioactivity. The percentage was determined by calculating

$$\frac{\text{Area}_{\text{peak I}} \text{ or } \text{Area}_{\text{peak II}}}{\text{Area}_{(\text{peak I} + \text{peak II})}} \times 100 = \%$$

[b] Total radioactivity was determined by summing the radioactive content in peaks I and II (which always equaled 100%, since these were the only major peaks; peak III in Figure 8 was ignored in this table due to its nonreproducibility). [c] The [^{14}C]glucosamine:[^{3}H]leucine ratio for each cell line was determined by the following equation for each peak.

$$\frac{\text{Area}_{[^{14}\text{C}]\text{glucosamine}}}{\text{Area}_{[^{3}\text{H}]\text{leucine}}} = \text{ratio}$$

lines was shown to possess at least two size classes of molecules which can be radiolabeled with both polysaccharide and polypeptide precursors.

Table IV shows the ratio of [^{14}C]glucosamine:[^{3}H]leucine in both peaks I and II for 3T3, SVT2, and revertant cells. From these ratios it can be determined that peak I of 3T3 SAM contained a larger proportion of carbohydrate than did the SAM from SVT2 cells. The SAM from the Con A revertant cells contained even less carbohydrate per amount of leucine-containing protein than either of the other two cell lines. The ratio of glucosamine:leucine in peak II of each cell line had comparable values. From the percentages of total radiolabel found in peak II from 3T3 (77.2% [^{14}C]glucosamine and 87.1% [^{3}H]leucine) the observation can be made that most of the radiolabeled SAM from this cell line is included in Sepharose 6B. Less of the total radioactivity was found in this second chromatographic peak from SVT2 and revertant cell lines. When comparing the ^{14}C:^{3}H ratios from peak I and peak II, it can be observed that peak II of 3T3 SAM contains much more of the total leucine radiolabel than the second peak from the other two cell lines.

To investigate the carbohydrate portion of these macromolecules the proteolytic enzyme pronase was utilized to digest the polypeptide away from the polysaccharide chains.

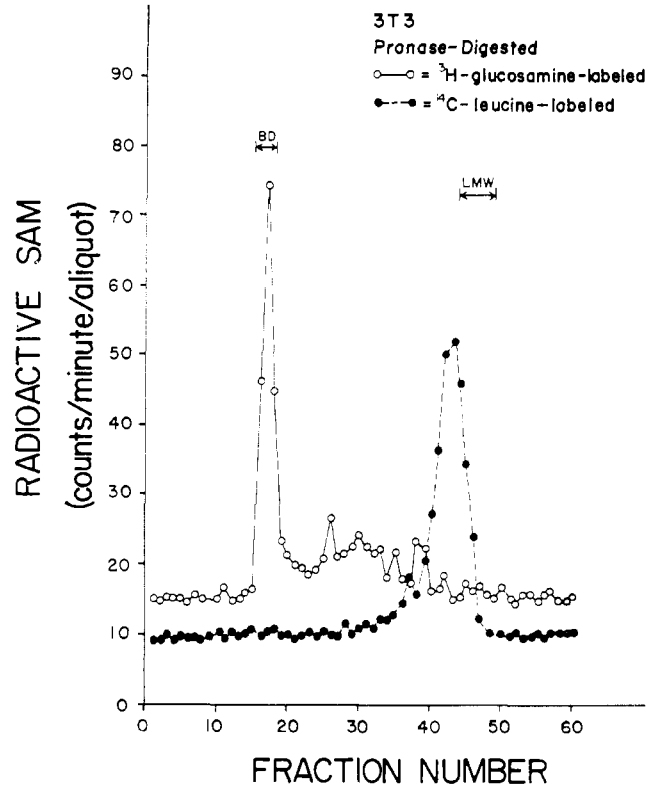

FIGURE 9: Chromatography of radiolabeled SAM from 3T3 cells on a Sephadex G-50 column subsequent to Pronase digestion. Radiolabeled SAM from 3T3 cells grown in [^{14}C]glucosamine and [^{3}H]-leucine was chromatographed on a Sephadex G-50 column after treatment with 1.0 mg of Pronase for 72 hr as described in the Materials and Methods section.

As can be seen from Figure 9 the previously excluded peak of [^{3}H]leucine radioactivity of 3T3 SAM was now eluting immediately ahead of the low molecular weight marker, thus indicating digestion of the leucine-radiolabeled material. The [^{14}C]glucosamine radiolabel, however, was still excluded from the resin. Identical results were obtained with Pronase digests of SVT2 SAM.

Figure 10 gives the elution profile from Sepharose 6B gel of glucosamine-radiolabeled SAM from both 3T3 and SVT2 cultures after Pronase digestion for 72 hr. Subsequent to Pronase digestion, the elution profile on Sepharose 6B (Figure 10) was altered in that a third peak (III) of radioactivity was found containing 32.4% of the total radioactivity in the 3T3 and 35.1% of the SVT2 total radioactivity (Table V). This lower molecular weight peak (peak III) of polysaccharide was apparently larger than the exclusion limit of G-50 (10,000) since it was not included using that chromatography system. SAM digestions from both normal and virus-transformed cells liberated this peak of radioactivity. Table V gives the percentages of total radioactivity in each peak and the ratio of the radioactivities contained in each peak. It can be determined that the radioactivity in the third peak could not have come from only one of the peaks of higher molecular weight. Since the ratios of peak I:peak II for the two cell lines are somewhat smaller subsequent to Pronase digestion, it would indicate that more radioactivity was lost from peak I than from peak II. After Pronase digestion the resulting ratio of the two peak sizes was 90.5% of the original value for SVT2, and 90.8% of the original 3T3 value.

To determine the size of the polypeptide portion of this material extensive enzymatic digestion of SAM by hyaluroni-

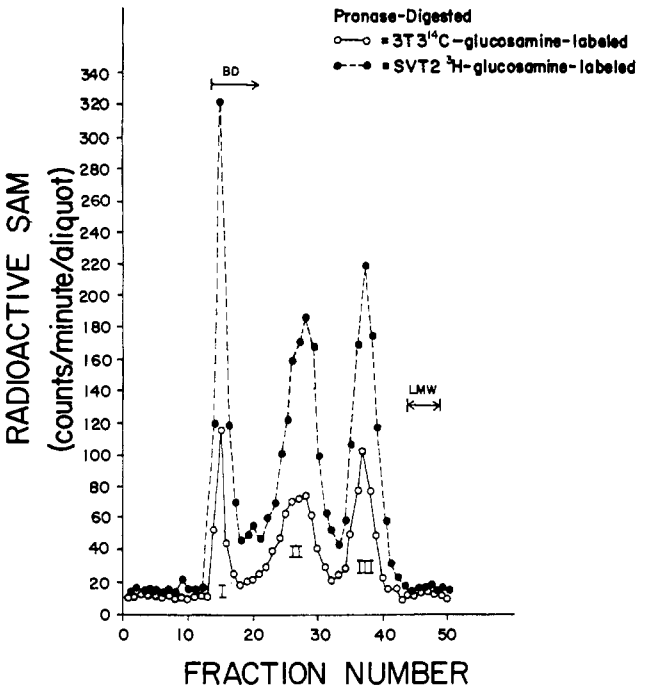

FIGURE 10: Sepharose 6B chromatography of glucosamine-radiolabeled SAM from 3T3 and SVT2 cells after Pronase digestion. Pronase digestion of SAM and Sepharose chromatography of glucosamine-radiolabeled SAM have been described in the Materials and Methods section and Figure 7.

dase were performed. SAM from both 3T3 and SVT2 cells was digested with hyaluronidase and chromatographed on Sephadex G-50 gel (Figure 11). In each digestion most of the [^{14}C]glucosamine radioactivity was found in a single peak of low molecular weight (see Table VI) indicating complete carbohydrate breakdown. [^{3}H]Leucine radioactivity was found in two peaks, the first being excluded from the gel and containing 31.7% of the total radioactivity from 3T3 SAM and 41.0% from SVT2 SAM. The included peak contained 68.3 and 59.0% of the total radioactivity of 3T3 and SVT2 preparations. Hyaluronidase is known to leave polysaccharide fragments still bound to the protein chain since its action removes sections of carbohydrate, and is not sequentially degradative in its action (Meyer, 1969). This remaining carbohydrate (see Table VI) would add to the molecular size of the polypeptide in the excluded region and may explain the sizable amount of excludable protein. On the other hand, SAM fractions may contain some polypeptide material which is large.

TABLE V: SAM Distribution Subsequent to Pronase Digestion and Sepharose Chromatography.[a]

	Percentage of Total Radioactivity[b] (%)			
	Self-Digestion[c]		Pronase Digested[d]	
	3T3	SVT2	3T3	SVT2
Peak I	33.6	40.0	20.4	25.4
Peak II	66.4	60.0	44.4	42.1
Peak III			35.1	32.4
Total	100	100	100	100
Peak I[e]:peak II	0.507	0.667	0.461	0.604

[a] The data refer to the recovery (in percentages) of radiolabeled material as seen in Figure 10 and from self-digestions carried out in the absence of Pronase as described in the Materials and Methods section. Peak I indicates the excluded peak of radioactivity in these figures. Peak II represents the higher molecular weight included peak of radioactivity seen in Figure 10. Peak III is the lowest molecular weight peak seen in Figure 10. [b] The percentage was determined by the following formula

$$\frac{\text{Area}_{\text{any peak}}}{\text{Area}_{(\text{peak I + peak II + peak III})}} \times 100 = \%$$

[c] From unpublished data. [d] See Figure 10 for procedures. [e] The ratio was determined by dividing the area of peak I by the area of peak II.

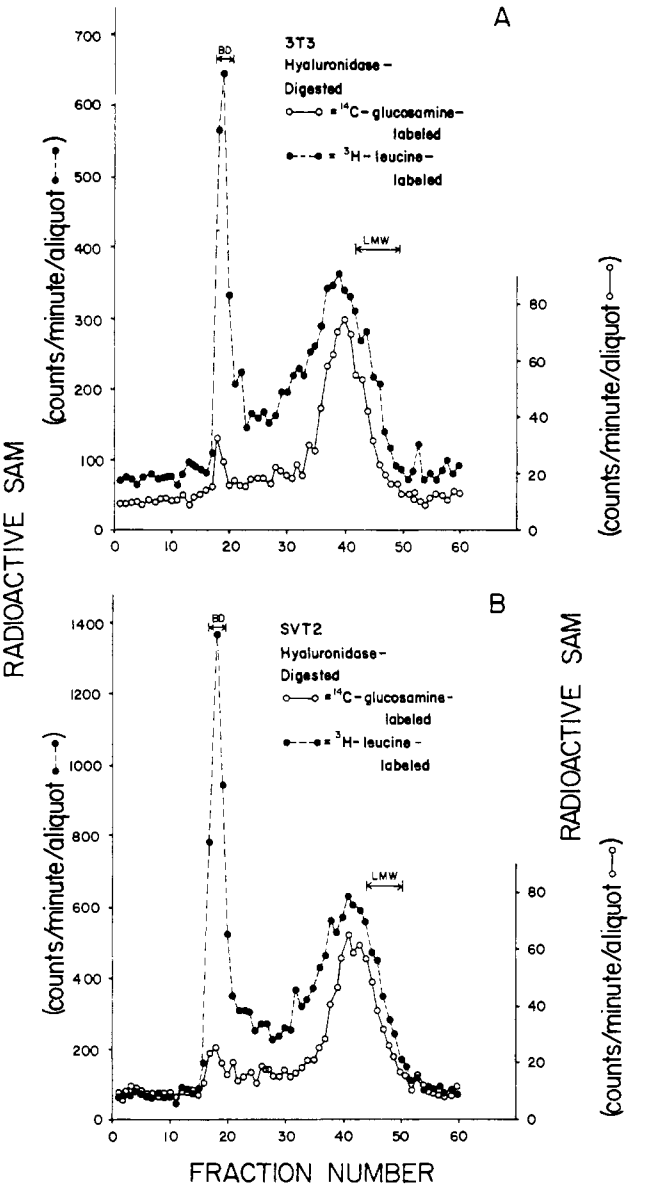

FIGURE 11: Sephadex G-50 chromatography of hyaluronidase-digested radiolabeled SAM. Hyaluronidase digestions were performed as described in the Materials and Methods section, with the exception that after 48-hr digestion, 700 additional units of hyaluronidase was added to ensure complete digestion by the end of the 72-hr treatment period. The resulting digestions of 3T3 SAM (A) and SVT2 SAM (B) were chromatographed on Sephadex G-50 resin as was previously described in Figure 2.

TABLE VI: Distribution of [^{14}C]Glucosamine and [^{3}H]Leucine Radiolabeled SAM Subsequent to Hyaluronidase Digestion and Sephadex Chromatography.[a]

	Percentage of Total Radioactivity[b] (%)			
	[^{14}C]Glucosamine		[^{3}H]Leucine	
	3T3	SVT2	3T3	SVT2
Peak I	13.3	14.2	31.7	41.0
Peak II	86.7	85.8	68.3	59.0
Total radioactivity	100	100	100	100

[a] Data refer to the recovery of radiolabeled material after hyaluronidase digestion as illustrated in Figure 11. Peak I represents the peak of radioactivity excluded from the resin, and the single included radioactive peak is identified as peak II. [b] See Table V for percentage determinations.

Further experiments will be necessary to define the nature of this excludable protein material.

Discussion

These experiments have examined the molecular composition of the substrate-attached material first identified by Culp and Black (1972b) from cultured mouse fibroblasts, both normal and virus transformed. It has been proposed that this material may be important in cell-to-substrate adhesion and may yield information on mechanisms of cell-to-cell adhesion, thereby explaining to some degree the phenomenon of contact inhibition of growth.

To determine if some, or all, of the SAM deposited by cells is glycosaminoglycan, a precipitation assay for GAG was developed based on the noted insolubility of these negatively charged macromolecules in solutions of cetylpyridinium chloride (Toole and Trelstad, 1971; Kraemer, 1971c). The assay was quantitative for precipitating and analyzing glycosaminoglycans, while causing minimal coprecipitation of large background levels of radioactive sulfate (Figure 1). There may be other negatively charged glycoproteins, such as sialylated mucins, which also coprecipitate with CPC. More study will be required to determine if CPC is specific for only GAG-type polysaccharides.

All of the glucosamine-radiolabeled SAM was precipitable with CPC, suggesting that it indeed was GAG. Approximately two-thirds of the material was precipitable with CCl_3COOH;[3] this material may be a subclass of GAG which contained covalently linked protein. Similarly, all SAM, which had been isolated from cells grown on radioactive glucosamine or sulfate eluted at high salt concentrations with nonsulfated GAG's such as hyaluronic acid from DEAE-Sephadex columns (Figure 3), was completely digested with hyaluronidase to small fragments (Figure 2; an indication that it was not of the heparin family of GAG (Meyer, 1969)), and failed to be radiolabeled with precursor sulfate. Essentially all of the sulfated GAG produced by normal or transformed cells was secreted into the medium and only very small amounts (always less than 0.5% of the total) were found in the cell fraction.

To prove that the SAM was hyaluronic acid, the amino sugar component of this material was shown to be glucosamine (Figures 4 and 5) and not galactosamine (the amino sugar component of nonsulfated chondroitin) (Kraemer, 1971c). SAM from normal or transformed cells was resistant to digestion with chondroitinase ABC, a property specifically characteristic of hyaluronic acid (Yamagata *et al.*, 1968), under conditions where sulfated and nonsulfated chondroitins were efficiently broken down to $\Delta^{4,5}$-unsaturated disaccharides. Thus, the SAM polysaccharide material from Balb/c 3T3 cells or SV40-transformed cells has been shown to be hyaluronic acid.

Recent evidence from several laboratories (Atkins and Sheehan, 1973; Dea *et al.*, 1973) indicates that hyaluronic acid in certain states (and possibly in aqueous solution) possesses a helical secondary structure. It is interesting to speculate that molecules of hyaluronic acid, when bound to the substrate, maintain a highly ordered structure which results in adhesion of cells by interaction with the cell surface in a highly ordered fashion. The binding of cells with a negatively charged surface to a "glue-like" layer of hyaluronic acid, which is also negatively charged, may be mediated by divalent calcium ions. This would explain the release of cells by a Ca^{2+}-specific chelating agent (EGTA), while leaving the SAM layer tightly bound to the substrate by some unknown mechanism. Perhaps a layer of this "glue" is also sandwiched between normal cells when they come into contact, preventing adjacent cells from crawling over each other and preventing further DNA synthesis by immobilizing the surface membrane of the cell (Abercrombie, 1967). A considerable amount of work will be required to determine if a layer of SAM is deposited between cells and what controls the production and deposition of this material between normal or virus-transformed cells.

Other evidence[3] indicates that SAM is directly involved in cell-to-substrate attachment, that it is deposited directly on the substrate by the cell and is not deposited from the large pool of medium-secreted glycoproteins and GAG, and that it may be important in determining cell morphology and growth properties.

With the use of enzyme digestions the polysaccharide and polypeptide components were examined. After digestion with Pronase to remove the major portion of polypeptide chains, the polysaccharide was shown to elute in three peaks from columns of Sepharose gel: an excluded peak (greater than one million molecular weight) and two included peaks (Figure 10). The lower molecular weight peak of these two was not found prior to Pronase treatment, while the included peak of larger-sized SAM exhibited a molecular size similar to that of the included peak of glycoprotein before proteolysis (Figures 7 and 10). This smallest molecular-sized peak was shown to be the result of carbohydrate released from both of the other peaks (Table V).

Upon hyaluronidase digestion to remove a major portion of the polysaccharide, it was found that there was at least two size classes of polypeptide (Figure 11). One class of small polypeptide chains, which may be covalently linked to hyaluronate polysaccharide chains because (1) 0.1% sodium dodecyl sulfate or 0.1 N NaOH was used to prevent aggregation and (2) this peak of radioactivity was not seen prior to hyaluronidase treatment, using enzyme free of any proteolytic activity.

The SAM from Balb/c 3T3 cells, SV40-transformed 3T3 cells, and Con A revertant variants of the transformed cells has been shown to be very similar. All of the polysaccharide material is hyaluronic acid, and most of the protein is relatively small and covalently linked to the hyaluronate. There are minor variations in the proportion of polysaccharide to

[3] L. A. Culp, manuscript in preparation.

protein in the two major peaks separable by Sepharose 6B chromatography from normal or transformed cells. Since all of the SAM studied in this paper was from cells which were sparsely seeded and growing exponentially, further experiments will be performed to determine if SAM is modified after normal or revertant cells become growth inhibited when the cultures have reached confluence.

Several laboratories have reported the presence of proteinaceous material between the cell and its substrate by a variety of methods. Revel and Wolken (1973) have observed these deposits by analysis of thin sections and replicas in the electron microscope using baby hamster kidney fibroblasts and mouse L cells. Rosenberg (1960) and Poste *et al.* (1973) using ellipsometry as a tool investigated the thickness and the rate of deposition of substrate-attached materials from a variety of normal, virus-transformed, and tumor cell lines. Maslow and Weiss (1972) established the deposition of ^{51}Cr-labeled surface material onto the substrate in cultures of mouse fibroblasts and Ehrlich ascites tumor cells.

The influence of various metabolic inhibitors upon attachment to the substrate of Swiss 3T3 or SV40-transformed 3T3 cells was studied by Kolodny (1972). Only low temperature and colchicine were effective in inhibiting cell attachment after trypsinization, while inhibitors of macromolecular syntheses were ineffective. This implicated some metabolic activity of the cell, such as microtubule assembly, in the attachment and spreading of fibroblasts.

Oppenheimer and Humphreys (1971) have identified a class of macromolecules from mouse teratoma cell cultures which are important in cell-to-cell adhesion and which could be released from cell aggregates with chelators of divalent cations. These materials have many of the properties established in our system: (1) highly negatively charged, (2) very large size ($>10^6$ daltons), and (3) released from cell aggregates with chelating agents. It will be interesting to determine (1) if the Oppenheimer–Humphreys aggregation factors are hyaluronic acid and (2) if hyaluronic acid is produced by normal and revertant cells in our system in response to cell-to-cell contact among growth-inhibited cells, as a mechanism for establishing strong adhesive forces between cells and thus preventing further cell movement and division.

Very few qualitative differences were found in SAM deposits between normal and transformed cells in this study, although much more information will be required to determine the fine structure of these materials. Perhaps the differences in cell spreading and strength of cell-to-substrate adhesion are primarily due to the amounts of SAM deposited and its topographical distribution.

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